Nephrol Dial Transplant (2002) 17 [Suppl 1]: 8–12

Neuroprotective properties of epoetin alfa

Anthony Cerami[1], Michael Brines[1], Pietro Ghezzi[1,2], Carla Cerami[1] and Loretta M. Itri[3]

[1]The Kenneth S. Warren Institute, New York, USA, [2]Department of Molecular Biochemistry and Pharmacology, Mario Negri Institute for Pharmacological Research, Milan, Italy and [3]Genta, Inc., Berkeley Heights, New Jersey, USA

Abstract

Erythropoietin and its receptor function as primary mediators of the normal physiological response to hypoxia. Erythropoietin is recognized for its central role in erythropoiesis, but studies in which recombinant human erythropoietin (epoetin alfa) is injected directly into ischaemic rodent brain show that erythropoietin also mediates neuroprotection. Abundant expression of the erythropoietin receptor has been observed at brain capillaries, which could provide a route for circulating erythropoietin to enter the brain. In confirmation of this hypothesis, systemic administration of epoetin alfa before or up to 6 h after focal brain ischaemia reduced injury by 50–75%. Epoetin alfa also limited the extent of concussive brain injury, the immune damage in experimental autoimmune encephalomyelitis and excitotoxicity induced by kainate. Thus, systemically administered epoetin alfa in animal models has neuroprotective effects, demonstrating its potential use after brain injury, trauma and multiple sclerosis. It is evident that erythropoietin has biological activities in addition to increasing red cell mass. Given the excellent safety profile of epoetin alfa, clinical trials evaluating systemically administered epoetin alfa as a general neuroprotective treatment are warranted.

Keywords: brain injury; encephalitis; epoetin alfa; erythropoietin; ischaemia; neuroprotection

Introduction

The most well-known action of erythropoietin is its haematological effect, whereby in response to hypoxia the kidney produces erythropoietin, which targets erythroid progenitor cells to increase the number of mature red blood cells, thereby increasing oxygen delivery [1]. The mechanism of this regulation of red blood cell production is primarily an anti-apoptotic effect on committed erythrocyte precursors, rather than a stimulation of growth or development [2,3]. Apoptosis may occur at any of several points along the pathway of development from erythrocyte precursors to mature erythrocytes in the bone marrow, e.g. as erythroid colony-forming units develop into pro-erythroblasts, or as pro-erythroblasts develop into reticulocytes. In the bone marrow, erythropoietin interacts with a specific receptor to up-regulate a family of genes known as *bcl-2*, which has been associated with inhibition of apoptosis of erythroid cells. This anti-apoptotic mechanism of erythropoietin appears to be important in other tissues, in which erythropoietin has been shown to operate.

Effects of erythropoietin in the central nervous system

Using available methods to examine erythropoietin production and receptors in other tissues, the brain was found to have an abundance of both. For example, in response to hypoxia, astrocytes produce erythropoietin, which then interacts with specific receptors on neurones and increases their tolerance to hypoxia [4]. Neurones themselves also produce erythropoietin in response to hypoxia [4]. The mechanism of tolerance to hypoxia in these brain cells can be presumed to be similar to that in bone marrow, i.e. an anti-apoptotic effect.

For many years, it was assumed that erythropoietin, a large glycoprotein, would not cross the blood–brain barrier, which is thought to be impenetrable to molecules >500 Da. Studies of cytokines, however, suggested that preferential transport across the blood–brain barrier may exist for some types of larger molecules [5], and erythropoietin is now known to be one such molecule. Immunocytochemical techniques have shown that the erythropoietin receptor can be found within and around brain capillaries in animals and humans. Transmission electron microscopy

Correspondence and offprint requests to: Anthony Cerami, The Kenneth S. Warren Institute, 712 Kitchawan Road, Ossining, NY 10562, USA. Tel: +1 914 762 7668, fax: +1 914 762 7445, e-mail: acerami@kswl.org

© 2002 European Renal Association–European Dialysis and Transplant Association

confirms that the predominance of anti-epoetin alfa receptor immunoreactivity is within astrocytic endfeet surrounding capillaries and within or on the surface of capillary endothelial cells [6]. These observations suggest an anatomical basis for direct transport of erythropoietin from the systemic circulation into the parenchyma of the brain across the blood–brain barrier in the absence of any neural insult. This hypothesis has been confirmed using biotinylated epoetin alfa in experimental animals [6]. At 5 h after systemic injection, biotinylated epoetin alfa was observed around capillaries and extending into the brain parenchyma, but not around the larger vessels, suggesting a specific transport mechanism. In addition, co-injection of excess amounts of unlabelled epoetin alfa significantly reduced the amount of biotin label, consistent with a specific and saturable transport mechanism. Also, epoetin alfa administered peripherally to rats appeared in the cerebrospinal fluid after ~1.5 h and peaks at ~1% of the peripheral concentration at 3.5 h post-administration (unpublished observations).

It has been shown that epoetin alfa injected directly into the parenchyma can prevent stroke damage in experimental models [7]. However, a more practical approach in a clinical context would be to determine whether systemically administered epoetin alfa can prevent brain damage. Four animal models of brain damage have been investigated in this way: a focal ischaemia model; a cortical impact injury model; an acute experimental allergic encephalitis model; and a kainate toxicity model [6].

Focal ischaemia (stroke) model

In the focal ischaemia (stroke) model in rats, permanent occlusion of the middle cerebral artery and homolateral carotid artery followed by a 1 h reversible occlusion of the contralateral carotid artery is used to produce an area of brain damage consisting of an ischaemic core (related to the permanent occlusion) surrounded by a penumbra of reperfusion damage (related to the reversible occlusion) [6]. The cells in the penumbra are induced to undergo apoptosis. After 24 h, the animals are sacrificed and the volume of damaged brain measured using computerized image analysis. Epoetin alfa, 5000 IU/kg intraperitoneally (i.p.), given 24 h before arterial occlusion, significantly reduced the volume of damaged brain by ~75% compared with control animals given saline [6]. The damage that remains despite pre-treatment represents primarily the ischaemic core. Furthermore, epoetin alfa could be given at any time up to 3 h after arterial occlusion and still provide protection against infarction (Figure 1) [6]. Even administration at 6 h after arterial occlusion provided ~50% protection, though by 9 h the therapeutic window was closed.

The anti-apoptotic nature of the protection afforded by epoetin alfa has been demonstrated very recently using TUNEL labelling, which identifies dead neurones. The number of TUNEL-positive neurones in the ischaemic penumbra after focal cerebral ischaemia was markedly reduced in the brains of animals who received epoetin alfa compared with control animals that received saline [8].

Cortical impact injury (blunt trauma) model

In the blunt trauma mouse model, a blow is delivered to the intact calvaria by a calibrated pneumatic piston. This mechanical insult delivered to the brain elicits elements of ischaemic, excitotoxic and inflammatory injury and, if severe enough, produces a cavitary lesion after 7–10 days [9]. The brain is fixed, sectioned and stained 10 days after injury. When the mice are treated with epoetin alfa, 5000 IU/kg i.p., starting 24 h before or 0, 3 or 6 h after the blow and continuing for a further 4 days once daily, the volume of injured brain

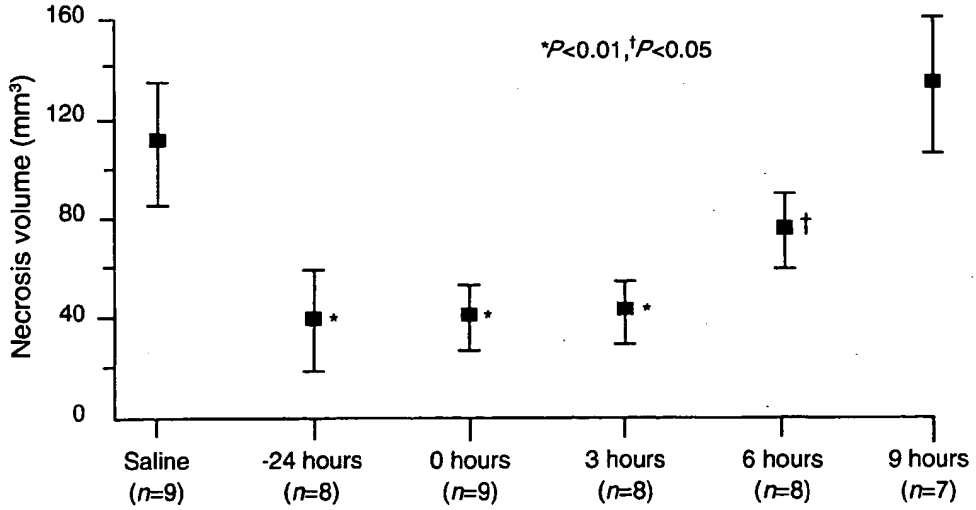
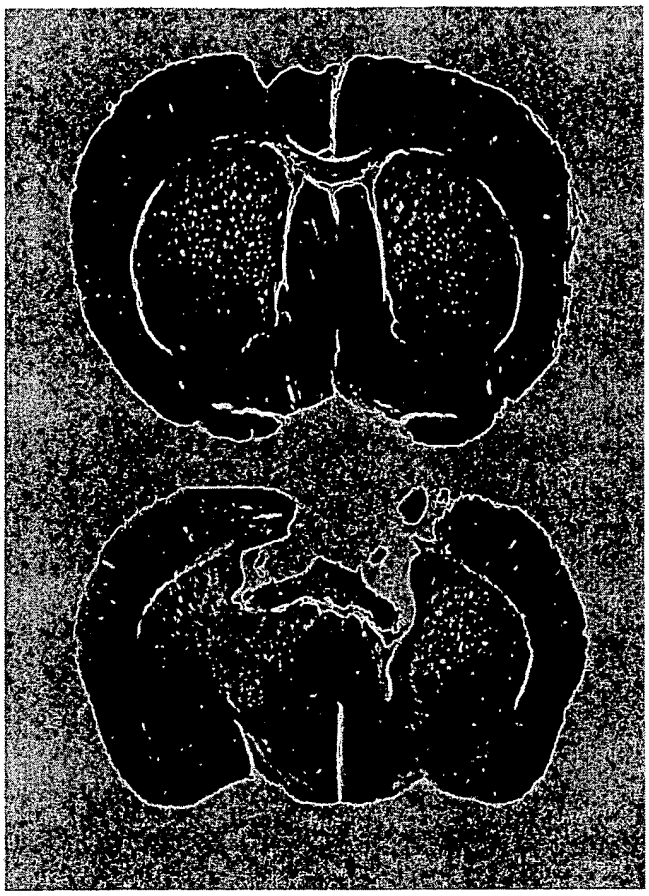


Fig. 1. Infarct volume after middle cerebral artery occlusion with or without epoetin alfa treatment. Male rats were given saline or epoetin alfa, 5000 IU/kg i.p., from 24 h before to 9 h after permanent middle cerebral artery occlusion with reversible carotid artery occlusion. (Reproduced with permission from [6].)



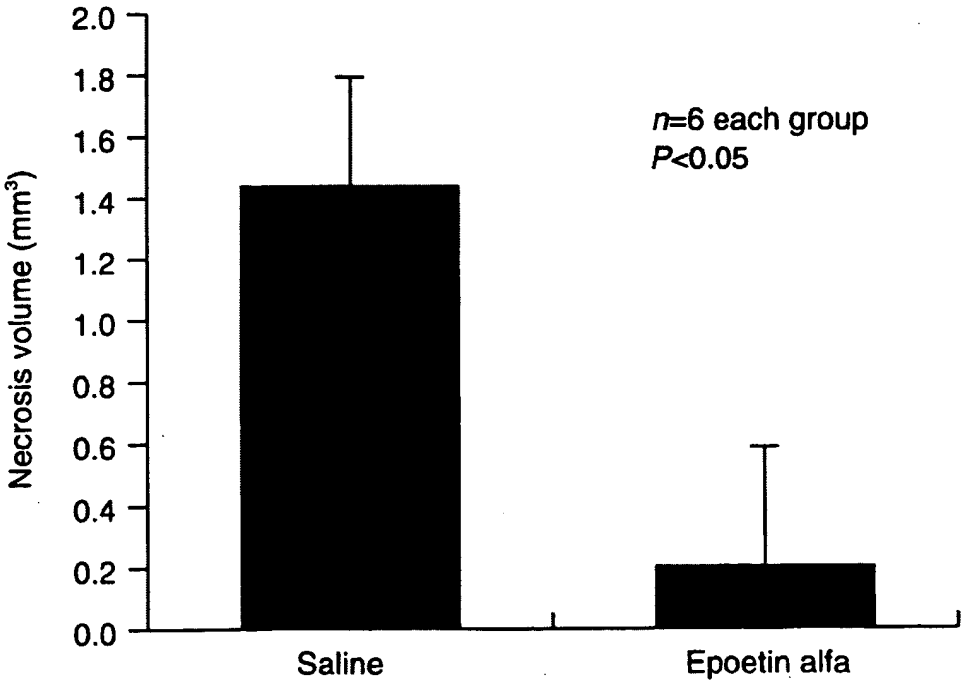


Fig. 2. Volume of cortical impact injury with systemic epoetin alfa treatment. Mice were given saline or epoetin alfa, 5000 IU/kg i.p., 24 h before blunt cortical impact from a calibrated piston. Epoetin alfa was continued for 4 days after injury. Extensive cavity necrosis was seen when examined 10 days after injury if treated with saline, in contrast to the minimal injury observed in the epoetin alfa-treated mice. (Reproduced with permission from [6].)

was significantly reduced by up to 90% compared with animals that received only saline (Figure 2) [6]. As in the focal ischaemia model, the therapeutic window extended to ~6 h after injury.

At a higher magnification, the necrotic core of the injury in animals treated with saline is seen to be surrounded by a large number of mononuclear inflammatory cells, which have migrated into the injured area and are probably responsible for much of the damage that occurs. In mice treated with epoetin alfa, however, very little inflammatory infiltrate was seen [6], indicating that epoetin alfa may also act as an anti-inflammatory agent in this type of injury. Thus, these experiments demonstrate the ability of systemically administered epoetin alfa to protect brain tissue from blunt trauma.

Acute experimental autoimmune encephalitis model

The reduction of the inflammatory response by epoetin alfa in the model of cortical impact injury suggests that epoetin alfa might be effective in other central nervous system (CNS) diseases where an inflammatory component is observed. This hypothesis was analysed in a rat model for acute experimental allergic encephalitis, an animal model for multiple sclerosis induced by immunization of animals with guinea-pig myelin basic protein and complete Freund's adjuvant [6]. This animal model is considered a typical inflammatory pathology of the CNS, as demonstrated by the protective action of several anti-cytokine molecules.

Immunized animals develop clinical symptoms within 10 days, which peak with an increasing degree of paralysis at day 12. Daily administration of epoetin alfa, 5000 IU/kg i.p. at day 3 after immunization for a period of 15 days, delayed the appearance of symptoms, and also significantly reduced the degree of paralysis compared with saline treatment (Figure 3) [6]. Studies for 3 weeks after epoetin alfa was discontinued showed no recurrence of symptoms in these animals, as is typically observed after discontinuing treatment with glucocorticoids or interferon-β [10]. However, the clinical manifestations observed with epoetin alfa in this model are consistent with anti-inflammatory agents such as glucocorticoids. These findings, together with those from the model of cortical impact injury, suggest that epoetin alfa has an anti-inflammatory action in inflammatory pathologies of the CNS.

Kainate toxicity model

Studies in cell culture suggested that apoptosis may be induced by conditions other than hypoxia, including addition of the glutamate analogue kainate [8]. Systemic administration of kainate in experimental animals increases brain excitotoxicity, which is a prominent feature of many forms of brain injury, causing seizures and, ultimately, death [11]. The effect of epoetin alfa on kainate-induced excitotoxicity was analysed in a toxicity model, in which the severity of seizures and time to death are measured in female mice.

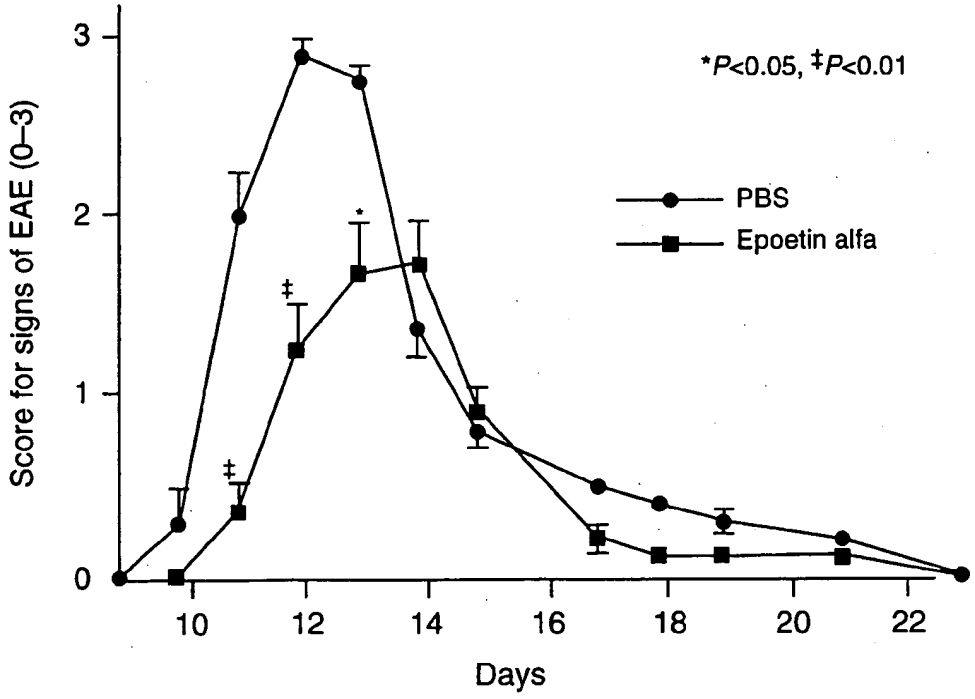


Fig. 3. Protective effect of epoetin alfa against acute experimental allergic encephalitis. Rats immunized with myelin basic protein and Freund's complete adjuvant received saline (PBS) or epoetin alfa, 5000 IU/kg/day i.p., starting 3 days after immunization. Epoetin alfa-treated rats show a delay in reduction of symptoms of experimental autoimmune encephalomyelitis (EAE). (Reproduced with permission from [6].)

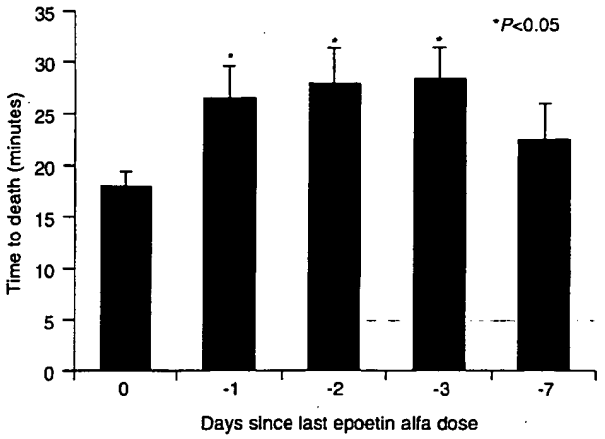


Fig. 4. Protective effect of epoetin alfa against death due to kainate-induced seizures. A single dose of epoetin alfa administered 0, 1, 2, 3 or 7 days before kainate 20 mg/kg i.p. administration continues to provide protection from kainate seizures for at least 3 days. (Modified from [6].)

Animals receiving epoetin alfa (5000 IU/kg, 24 h before administration of kainate) exhibited a significant reduction in mortality by ~45% compared with control animals and a significant increase in mean survival time of 42%. With lower doses of kainate that did not cause death, the severity of seizures was markedly less than in control animals. A single dose of epoetin alfa provided protection for at least 3 days (Figure 4). However, epoetin alfa did not protect against kainate toxicity if it was given 30 min before kainate or after the development of seizures. In contrast, conventional anti-epileptic agents can protect against kainate toxicity when given at the same time as or slightly after kainate administration, but protection depends on their continued presence [6]. These findings suggest that the protective effect of epoetin alfa on excitotoxicity is based on a different mode of action compared with conventional anti-epileptic agents, and presumably involves activation of gene expression that continues protection even in the absence of the cytokine [6].

Therapeutic potential of epoetin alfa in the central nervous system

The experimental findings described above suggest that epoetin alfa may have many potential therapeutic uses, particularly in the CNS. These include stroke, trauma (including surgery and radiotherapy), epilepsy and neurodegenerative diseases. Given the excellent safety profile of epoetin alfa, clinical trials are warranted to determine whether these experimental findings can be translated into therapeutic effects in humans.

References

1. Hillman RS, Finch CA. *Red Cell Manual*, 7th edn. FA Davis Company, 1996
2. Gregory T, Yu C, Ma A, Orkin SH, Blobel GA, Weiss MJ. GATA-1 and erythropoietin cooperate to promote erythroid cell survival by regulating bcl-x_L expression. *Blood* 1999; 94: 87–96
3. Silva M, Benito A, Sanz C *et al.* Erythropoietin can induce the expression of Bcl-x_L through Stat5 in erythropoietin-dependent progenitor cell lines. *J Biol Chem* 1999; 274: 22165–22169
4. Bernaudin M, Bellail A, Marti HH *et al.* Neurons and astrocytes express brain EPO mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain. *Glia* 2000; 30: 271–278
5. Pardridge WM. Drug delivery to the brain. *J Cereb Blood Flow Metab* 1997; 17: 713–731
6. Brines ML, Ghezzi P, Keenan S *et al.* Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci USA* 2000; 97: 10526–10531
7. Sakanaka M, Wen T-C, Matsuda S *et al. In vivo* evidence that erythropoietin protects neurones from ischemic damage. *Proc Natl Acad Sci USA* 1998; 95: 4635–4640
8. Sirén A-L, Fratelli M, Brines M *et al.* Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc Natl Acad Sci USA* 2001; 98: 4044–4049
9. Dixon CE, Lighthall KW, Anderson TE. Physiologic, histopathologic and cineradiographic characterization of a new fluid-percussion model of experimental brain injury in the rat. *J Neurotrauma* 1988; 5: 91–104
10. Van der Meide PH, De Labie MC, Ruuls SR *et al.* Discontinuation of treatment with IFN-beta leads to exacerbation of experimental autoimmune encephalomyelitis in Lewis rats. Rapid reversal of the anti-proliferative activity of IFN-beta and excessive expansion of autoreactive T cells as disease promoting mechanisms. *J Neuroimmunol* 1998; 84: 14–23
11. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* 1999; 22: 391–397

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Brain Research 1000 (2004) 19–31
BRAIN RESEARCH
www.elsevier.com/locate/brainres

Review

Erythropoietin and the nervous system

Sermin Genc[a,*], Tolga F. Koroglu[b], Kursad Genc[c]

[a] Department of Medical Biology and Genetics, School of Medicine, Dokuz Eylul University, Inciralti, 35340, Izmir, Turkey
[b] Department of Pediatrics, School of Medicine, Dokuz Eylul University, Inciralti, 35340, Izmir, Turkey
[c] Department of Physiology, School of Medicine, Dokuz Eylul University, Inciralti, 35340, Izmir, Turkey

Accepted 4 December 2003

Abstract

Erythropoietin (Epo) is a hematopoietic growth factor and cytokine which stimulates erythropoiesis. In recent years, Epo has been shown to have important nonhematopoietic functions in the nervous system. Nonerythropoietic actions of Epo include a critical role in the development, maintenance, protection and repair of the nervous system. A wide variety of experimental studies have shown that Epo and its receptor are expressed in the nervous system and Epo exerts remarkable neuroprotection in cell culture and animal models of nervous system disorders. In this review, we summarize the current knowledge on the neurotrophic and neuroprotective properties of Epo, the mechanisms by which Epo produces neuroprotection and the signal transduction systems regulated by Epo in the nervous system.
© 2004 Elsevier B.V. All rights reserved.

Theme: Development and regeneration
Topic: Neurotrophic factors: biological effects

Keywords: Erythropoietin; Neuroprotection; Neurodegeneration; Apoptosis; Ischemic preconditioning; Signal transduction

1. Introduction

Erythropoietin (Epo) was first identified as a hematopoietic cytokine acting as a survival and differentiation factor [43]. It is a 34-kDa glycoprotein that functions as a main regulator of erythropoiesis. During fetal development, Epo is initially produced in the liver, but shortly after birth, Epo production is subsequently shifted to the kidney [43]. Circulating Epo binds to its receptor expressed on erythroid precursor cells in bone marrow and results in the stimulation of erythropoiesis. The role of Epo in hematopoiesis is inhibiting apoptosis of erythroid precursor cells and supporting their proliferation and differentiation into normoblasts [43]. The induction of erythropoiesis results in a progressive improvement in the supply of oxygen to tissue. In addition, tissue oxygen supply functions as a critical hormonal feedback mechanism and a significant modulator of oxygen-dependent Epo production and erythropoiesis.

Epo was isolated from the urine of patients with aplastic anemia and Epo DNA probes were constructed from tryptic fragments of the isolate and the gene cloned in 1985 [43]. Soon, recombinant Epo became available and underwent evaluation in clinical trials for patients with end-stage renal disease. The US Food and Drug Administration approved Epo use in 1989, and it is now widely used for the treatment of anemia associated with renal failure, cancer, prematurity, chronic inflammatory disease and human immunodeficiency virus infection [43].

Epo was thought to be exclusively produced in fetal liver and adult kidney. However, several lines of evidences suggest that Epo and erythropoietin receptor (EpoR) are expressed by other tissues, including the nervous system. Different cell types (neurons, glial cells and endothelial cells) in the nervous system produce Epo and express EpoR. Epo has tissue-specific regulation and multiple actions in the nervous system. Epo and EpoR expression change significantly during brain development, thus indicating the importance of Epo in neurodevelopment. The cytokine Epo has been shown to possess neuroprotective characteristics following ischemic, hypoxic, metabolic, neurotoxic and excitotoxic stress in the nervous system. Epo acts in a coordinated fashion at multiple levels in the nervous system, including limiting the production of tissue-injuring molecules such as reactive oxygen species and glutamate, mod-

* Corresponding author. Tel.: +90-232-412-46-04; fax: +90-232-259-05-41.
E-mail address: sermingenc@hotmail.com (S. Genc).

0006-8993/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2003.12.037

ulation of neurotransmission, reversal of vasospasm, stimulation of angiogenesis, attenuation of apoptosis, modulation of inflammation and recruitment of stem cells. In this respect, the present review focuses on evidence that suggests Epo has neurotrophic and neuroprotective properties in the nervous system. Several mechanisms by which Epo produces neuroprotection and signal transduction systems regulated by Epo in the nervous system are also outlined.

2. Role of erythropoietin in neurodevelopment

Epo and its receptor have been identified in specific areas of the embryonic, fetal and adult brain of rodents, nonhuman primates and humans. The presence of Epo and its receptor in the developing brain suggests that these molecules play a role in neurodevelopment. Epo is a general morphogen and inducer of neurogenesis during early development [103,109]. During development, Epo is produced primarily in the fetal liver and is necessary for embryonic survival. Embryos that have deleted or nonfunctional Epo or Epo receptors die by embryonic day 13 due to defective erythropoiesis in the fetal liver. These mice have other organ abnormalities, such as ventricular hypoplasia and increased apoptosis in fetal liver, heart and brain. Recently, it has been shown that these defects can be corrected in animals expressing the EpoR transgene [108]. This finding suggests the importance of the Epo/EpoR system during ontogenesis. Further support for Epo as a neurotrophic agent during development comes from studies of EpoR knockout mice. In the absence of EpoR function during embryogenesis, there are a reduction in the number of neural progenitor cells, decreases in neurogenesis and increased neuronal apoptosis compared with wild-type controls. Cultures of cortical neurons from these knockout animals also exhibit increased sensitivity to low oxygen tension [109].

3. Expression of erythropoietin and erythropoietin receptor in the nervous system

It was previously thought that Epo function was unique to the hematopoietic system; however, recent studies have located functional EpoRs in a wide variety of organs, tissues and cells in animal models and in the human. These nonhematopoietic cells include endothelial cells [4,79], enterocytes [46,75], muscle (skeletal, smooth and cardiac) [68,74,99,103], placental tissues [84], insulin-producing cells [35] and neuronal and nonneuronal cells of the nervous system. Because Epo functions via activation of specific EpoR, evaluation of the distribution of EpoR helps in the understanding of the biological effects of Epo in the central nervous system (CNS). Both Epo and EpoR are functionally expressed in the nervous system of rodents, primates and humans. Differences in the structure, function and regulation of brain-derived Epo and its receptor compared with their somatic counterparts have been identified [65]. When brain Epo was compared with serum Epo, brain Epo was smaller in size and more active in vitro at low ligand concentrations. These differences appear to be caused by the different extent of sialylation.

3.1. In vitro neuronal and glial expression of erythropoietin and erythropoietin receptor

EpoR has been identified on several neuronal cell lines as well as in primary cultures of neurons and glial cells. Neuronal expression of Epo has been demonstrated first in PC12 rat pheochromocytoma and SN6 septal cholinergic cell lines [64]. Astroglial Epo production has also been verified in vitro [65]. EpoR expression has been found on neuronal cells [69,96]. In cultured cortical neurons of rats, the expression of EpoR has been demonstrated by immunostaining and reverse transcriptase-polymerase chain reaction (RT-PCR) [70]. Cytochemical immunostaining has revealed the expression of Epo and its receptor also in rat hippocampal neurons [24]. Epo and EpoR messenger RNA (mRNA) are expressed in mixed primary cultures of neural tissues and NT2 and hNT human neuronal cell lines [48]. Epo was detected by ELISA in media removed from mixed neuronal–glial cell cultures, and immunohistochemical staining confirmed the presence of EpoR on neurons and their supporting cells [48]. Epo mRNA expression and Epo production have also been demonstrated in cultured human astrocytes [72]. EpoR mRNA expression was found in cultured human neurons, astrocytes and microglia, but not in oligodendrocytes [72]. In contrast to human oligodendrocytes, rat oligodendrocytes express Epo and EpoR mRNA and proteins in vitro [95]. These differences may result from in vitro culture conditions or different species.

3.2. In situ studies showing the expression of Epo and EpoR in the nervous system

Epo and its receptor have been localized in specific areas of the embryonic, fetal and adult brain of rats, monkeys and humans. Binding studies on adult mouse brain sections revealed defined binding sites for radioiodinated Epo in distinct brain areas [30]. Major Epo binding sites were observed in the hippocampus, capsula interna, cortex and midbrain areas. mRNA encoding both Epo and EpoR have been detected in mouse brain by RT-PCR [30]. In primates, mRNA of Epo and the EpoR gene are present in the hippocampus, amygdala and temporal cortex of the primate brain [63]. Epo and EpoR mRNA have been identified in biopsies of the temporal cortex, the hippocampus, cerebellum and amygdala in human brain [63]. Spinal cord tissues of first- and mid-trimester human fetuses express EpoR mRNA [48,59]. The distribution of Epo and EpoR in the CNS changes as gestational age increases. EpoR transcripts in brain decrease during development falling by birth to less than 1–3% of the level in hematopoietic tissue [60]. Undif-

ferentiated neuroepithelial cells in human embryos express both Epo and EpoR [49]. The expression pattern becomes more distinct at later developmental stages with EpoR expression in astrocytes and Epo expression in neurons of the late fetal brain [49]. The presence of Epo and its receptor in developing brain, and their persistence in the mature brain, suggest that they play a role in both neurodevelopment and brain homeostasis [29,47].

In the adult human brain, only a weak expression of Epo and its receptor has been reported in neurons and astrocytes [49,91]. Similar to many brain regions examined, the frontal cortex and hippocampus exhibited intense immunoreactivity for EpoR in many medium to large neurons, but in a pattern restricted to the somata and proximal dendrites, and capillaries, particularly within the white matter [11]. Certain capillaries, especially within white matter, prominently express EpoR [11]. Both the endothelial cells and specialized astrocyte that completely invests the brain side of the capillary with end-feet possess abundant cytoplasmic and surface membrane EpoR protein. Transmission electron microscopy confirmed that the predominance of anti-EpoR immunoreactivity was located within the astrocytic end-feet surrounding the capillaries. In addition, substantial EpoR immunoreactivity was observed within or on the surface of capillary endothelial cells [11].

Immunohistochemistry performed using adult human spinal cord sections showed abundant EpoR immunoreactivity of capillaries, especially in white matter, and motor neurons within the ventral horn [19]. Epo immunoreactivity occurred within the neuropil surrounding motor neurons in a pattern consistent with dendrites. The distribution of Epo and EpoR in the spinal cord appears therefore to be of an autocrine/paracrine pattern.

Epo and EpoR are also produced locally in cells of the adult rat peripheral nervous system. EpoR has been identified in some axons and neuronal cell bodies in the dorsal root ganglion (DRG) cells, endothelial cells and Schwann cells of normal nerve [15]. The presence of Epo and EpoR in axons of normal nerve suggests that they are integral components of neuronal function.

4. Regulation of the Epo and EpoR expression in the nervous system

4.1. Developmental changes

Epo and EpoR expression change significantly during brain development, thus indicating the importance of the Epo/EpoR system in neurodevelopment. High EpoR expression has been found in embryonic mouse neural tissue and brain, reaching levels similar to that observed in adult bone marrow [61]. This expression decreases significantly during development and maturation of the brain, by up to 100-fold after birth [60]. The level of Epo in the human nervous system is also variable, with elevated production during gestation and reduced production after birth [49].

4.2. Oxygen-dependent expression of Epo/EpoR in the nervous system

Epo gene expression in most tissues, including brain, is regulated by hypoxia-inducible factor-1 (HIF-1) that is activated by a variety of stressors, including hypoxia [43]. The inducibility of Epo gene expression is tissue-specific with the strongest effect in kidney and brain, whereas in the uterus, Epo mRNA is only induced in the presence of estrogen [22]. Thus, Epo expression appears to be regulated in a tissue-specific manner, endorsing the tissue-specific functions of Epo. It has been demonstrated that astroglial expression of Epo is greatly enhanced by hypoxia at the level of mRNA [64]. Both Epo and EpoR mRNAs are inducible by hypoxia in hippocampal neuronal cultures [58]. The mRNA expression of Epo in cultured mouse neurons is greatly enhanced by hypoxia and it is completely prevented by cycloheximide, a protein synthesis inhibitor [8]. Thus, an oxygen sensing system, similar to that identified in hepatoma cell lines and the kidney, may control Epo production in both astrocytes and neurons. Desferrioxamine and $CoCl_2$, two agents known to mimic the hypoxic induction of Epo in hepatoma cells, can induce Epo mRNA expression in astrocytes and neurons [8]. Therefore, as with Epo production in the kidney and fetal liver, the effect of hypoxia on Epo production in neuronal cells is regulated in part via the transcriptional activator HIF-1. Hypoxia stimulates EpoR mRNA expression also in vivo. Expression of Epo and EpoR mRNA is evident in the hippocampus of rats exposed to hypoxia [58,90]. The expression of Epo and EpoR mRNA in the brain is increased after exposure to low oxygen levels [63]. The timing of Epo up-regulation in the brain is different than in the kidney; Epo mRNA expression peaks 2 h after the onset of continuous hypoxia in murine kidneys, decreasing to 30% of the maximum level by 8 h. In contrast, brain Epo mRNA expression peaks 4 h after hypoxic stimulation and remains high for 24 h [22].

4.3. Other factors influencing the Epo and EpoR expression in the nervous system

Hypoxia may not be the only relevant stimulus for brain Epo production. Other metabolic disturbances, such as hypoglycemia or strong neuronal depolarization that generate mitochondrial reactive oxygen species, may increase cerebral Epo expression through HIF-1 activation [20]. In human EpoR transgenic mice, anemic stress induces expression of the transgene and endogenous EpoR gene in hematopoietic tissue and brain [23].

Epo mRNA expression by cultured astrocytes is stimulated by insulin and insulin-like growth factors in a dose-dependent manner. Insulin and insulin-like growth factors are abundantly expressed in the CNS, but the physiological

importance of their stimulatory effect on Epo production in the brain is not known [66]. In vitro Epo expression by astrocytes is down-regulated after exposure to inflammatory cytokines such as interleukin-1β, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [72].

4.4. Altered expression of Epo and EpoR after nervous system injury

The induction of both Epo and EpoR gene expression by hypoxia suggest that Epo could act on the CNS as a neurotrophic and neuroprotective factor, particularly in conditions of neural damage, such as hypoxia, ischemia or brain hemorrhage. Marked changes in Epo and EpoR gene expression have been reported to occur in brain tissue after ischemic injury [7,82]. An induction of EpoR gene expression in the ischemic penumbra after middle cerebral artery occlusion has been reported [82]. Bernaudin et al. [7] described, for the first time, a temporal and spatial cellular expression of Epo and EpoR with the evolution of a cerebral infarct after focal permanent ischemia in mice. In addition to a basal expression of Epo in neurons and astrocytes, a post-ischemic Epo expression has been localized specifically to endothelial cells (1 day), microglia/macrophage-like cells (3 days) and reactive astrocytes (7 days after occlusion) [7].

In normal human brain, weak Epo/EpoR immunoreactivity is mainly neuronal [91]. Expression of EpoR is up-regulated in blood vessels, neurons and astrocytes following acute ischemic injury in human brain and remains increased in reactive glia in older ischemic infarcts (>18 days after stroke) [91]. In fresh cerebral infarcts, Epo immunoreactivity appears in vascular endothelium and EpoR in microvessels and neuronal fibers. In older infarcts, reactive astrocytes exhibit Epo/EpoR immunoreactivity. Acute hypoxic brain damage is associated with vascular Epo expression. The pronounced up-regulation of Epo/EpoR in human ischemic/hypoxic brains underlines their role as an endogenous neuroprotective system [91]. Other stressors, such as epileptic seizures, also dramatically stimulate up-regulation of EpoR within the cerebral microvasculature [10].

Epo expression is up-regulated in the rat sciatic nerve, particularly in Schwann cells after painful chronic constriction injury neuropathy model [15]. In contrast to this, in crush injury model having many TUNEL-labeled DRG cell bodies and mechanical hyperalgesia, EpoR was down- regulated in small and large mechanosensitive neurons [15].

5. Neuroprotective effect of erythropoietin in the nervous system

Recombinant human Epo (rhEpo) treatment has a beneficial effect on brain function, as demonstrated by findings from neuropsychological tests and electrophysiological evaluation [73]. This improvement is correlated with a progressive increase in the hematocrit level and therefore with the correction of the hypoxic state of the brain. If anemia is corrected, and the hematocrit levels return to normal, a further improvement in neurocognitive function has been reported [76]. However, several studies in vitro and in vivo have shown that Epo could also have a direct neurotrophic and neuroprotective effect.

5.1. In vitro studies

Epo has been recognized as the main regulator of erythropoiesis and was thought to be exclusively produced in fetal liver and adult kidney. However, there is growing evidence that Epo, like other hematopoietic factors, is also implicated in the CNS. Some hematopoietic factors, including Epo, act on central cholinergic neurons as neurotrophic factors, influencing their differentiation, survival and regeneration [53]. Epo increases the choline-acetyltransferase activity of the septal cholinergic cell line SN6 as well as in primary cultured septal neurons in vitro [96]. Epo rapidly increases the cytosolic concentration of free calcium, induces membrane depolarization, increases dopamine release, nitric oxide (NO) production and tyrosine hydroxylase activity and increases cell viability of differentiated PC12 cells in the absence of serum and nerve growth factor [54,64]. This increased viability is dose dependent, Epo specific and calcium mediated, as it is abolished by anti-Epo antibody and nicardipine [54]. Epo protects primary cultures of neurons from glutamate neurotoxicity in a dose-dependent manner and maximal protection occurs when cells are pretreated for 8 h, suggesting RNA and protein synthesis are necessary for this protection [70]. Moreover, in these neuronal cells, Epo produces a rapid and transient increase in intracellular calcium that is necessary for neuroprotection because blockade of calcium influx blocks these effects. Specificity and biological relevance in this system have been demonstrated by the observation that neutralization of endogenous Epo with soluble EpoR prevents Epo-induced protection against glutamate toxicity [70].

A specific neurotrophic sequence of 17 amino acids within the Epo molecule has been identified which triggers differentiation, increases choline-acetyltransferase activity and prevents cell death in both murine NS20Y and human SK-N-MC neuroblastoma cell lines, but does not promote the proliferation of erythropoietic cell lines or mouse primary spleen cells [16]. This activity is blocked by the addition of an antibody to the extracellular domain of the EpoR, indicating that the effects are EpoR mediated. In a model of cerebral ischemia in vitro, comprising hypoxia and glucose deprivation, Epo protects cultured neurons, but not astrocytes, from death [81,88]. Epo treatment of cultured hippocampal neurons from newborn rats at the onset of hypoxia can prevent neuronal death [58]. Epo shows similar effects in human neuroblastoma cells [48]. The viability of primary rodent embryonic cortical neurons was further increased by Epo stimulation [109].

The promoting effect of Epo on the differentiation and maturation of rat oligodendrocytes has been reported [95]. These findings suggest that Epo might affect the ability of oligodendrocyte lineage cells to promote myelin repair in the normal and damaged adult CNS.

5.2. In vivo studies

Multiple models of nervous system injury (mouse, rat, gerbil and rabbit) have been used to demonstrate the effectiveness of Epo as a neuroprotective agent, including focal and global cerebral ischemia [7,11,14,18,34,82,83], experimental autoimmune encephalomyelitis (EAE) [1,11], kainic acid-induced seizures [11], experimental traumatic brain injury [11], neurotoxin-induced experimental Parkinsonism [37], neonatal hypoxic–ischemic brain injury [55], subarachnoid hemorrhage (SAH) [2,40], spinal cord ischemia [19] and injury [34,39,50], retinal ischemia [41,45] and peripheral nerve injury [34,42]. Epo prolongs the survival of septal neurons in rats with cholinergic-septal neuronal lesions produced by fimbria–fornix transactions in vivo [96]. When Epo was locally injected into mice, the frequency of motor end plate sprouting in adjacent muscles increased [16]. To varying degrees, all studies have shown that Epo treatment decreases the structural damage resulting from experimental brain injury and, where investigated, improves neurological function.

5.2.1. Cerebral and spinal ischemia models

Many experimental studies have shown that Epo has a neuroprotective effect during cerebral ischemia. Intraventricular administration of rhEpo preserved place navigation, limited the degree of cortical infarction and supported neuronal survival in the thalamus of stroke-prone spontaneously hypertensive rats with permanent occlusions of the middle cerebral artery [82]. Epo ameliorated neuron survival in an experimental model of cerebral ischemia in the gerbil [83]. Infusion of Epo into the lateral ventricles of gerbils prevented ischemia-induced learning disability and rescued hippocampal CA1 neurons from lethal ischemic damage. Specificity and biological relevance in this study have been demonstrated by the observation that neutralization of endogenous Epo with soluble EpoR augments ischemic brain damage in vivo [83]. It thus appears that endogenous Epo plays a critical role in neuronal survival after ischemic injury. In a similar study, treatment with Epo significantly reduced delayed neuronal death in the CA1 area of the hippocampus and prevented cognition impairment in the passive avoidance test. These data indicate that Epo neuroprotective effects in brain ischemia are associated with the preservation of learning function [18]. Systemic administration of Epo also significantly reduced hippocampal CA1 neuronal loss in the gerbil model [14]. Intraventricular injection of recombinant mouse Epo significantly reduced infarct volume in mice with focal permanent cerebral ischemia induced by permanent occlusion of the left middle cerebral artery [7]. These studies confirm that Epo plays a role in the brain response to ischemia, and it may be of therapeutic value in patients with stroke [33]. It is important to establish whether or not systemically administered rhEpo can cross the blood–brain barrier (BBB) since intrathecal administration is not a practical approach in most clinical settings. In a recent study, it was demonstrated that systemically administered rhEpo crossed the BBB and significantly reduced tissue damage in an ischemic stroke animal model, even when it was administered 6 h after the induction of stroke [11]. Although the BBB is considered impermeable to large molecules, recent studies clearly demonstrate that some high-molecular mass molecules can be specifically transported into the brain across the capillary endothelium. Two different types of EpoR have been found in the cerebral capillary endothelium [104]. It has therefore been suggested that circulating Epo could bind to these receptors, which are present on the luminal surfaces of the endothelial cells. This would initiate endocytosis, followed by translocation into the brain. If so, systemically administered rhEpo could directly reach the brain, providing protection against brain injury. Early in vivo studies testing Epo as a neuroprotective agent administered Epo intraventricularly in order to bypass the BBB. The ability of Epo to penetrate the BBB is pivotal to its application as a neurotherapeutic agent in clinical settings because intrathecal administration is impractical. When given at doses appropriate for erythropoiesis (200–400 U kg^{-1} per dose), Epo does not cross the BBB in detectable amounts [47]; however, when given in suprapharmacological doses (2000–5000 U kg^{-1} per dose), Epo can cross to rat CNS as demonstrated by biotinylated Epo [11]. High-dose Epo, when given systemically, crosses the intact BBB, producing cerebrospinal fluid concentrations ranging from 50 to 350 mU ml^{-1} at 3–3.5 h following injection in adult rodents and nonhuman primates [47]. The pharmacokinetics of systemically administered Epo under conditions of CNS injury and disruption of the BBB should be clarified.

Additional evidence has shown widespread efficacy of rhEpo in injury models of the spinal cord. Both an acute as well as a delayed beneficial action of systemically administered Epo has been reported in rabbit ischemic spinal cord injury [19].

5.2.2. Subarachnoid hemorrhage model

The neuroprotective effect of Epo was observed in experimental SAH. Epo administration immediately after experimental SAH effectively reduced the mortality rate and enhanced functional recovery [40]. Epo has a neuroprotective effect in SAH because it can attenuate acute vasoconstriction and prevent brain ischemic damage that frequently follows SAH. Intraperitoneal administration of rhEpo at a dose of 1000 U kg^{-1} significantly reversed the vasoconstriction of the basilar arteries and decreased the amount of necrotic neurons compared with untreated animals [2,40]. Epo may reverse acute vasoconstriction and reduce ischemic neuronal damage by enhancing the endothelial release of

NO during the early stages of SAH. An acute decrease in cerebral NO levels after SAH has been reported [85]. Epo increases NO synthase (NOS) activity in neuronal and endothelial cells [6,54]. Moreover, rhEpo may act directly on cerebral arteries by binding to specific endothelial receptors, thus exerting a competitive effect in the control of the cerebrovascular tone [12]. In fact, a single subcutaneous bolus of Epo normalizes cerebral blood flow autoregulation after SAH in rats [93].

5.3. Experimental autoimmune encephalomyelitis model

The effect of systemic Epo on the inflammatory component of acute and chronic EAE has been evaluated in Lewis rats [1,11]. In acute EAE, administration of Epo intraperitoneally and daily from day 3 after immunization with myelin basic protein delayed the onset of EAE and decreased its clinical score at peak time (days 12–13). In this model, Epo markedly diminished inflammation, delayed the increase of TNF-α levels, without altering their peak levels, and markedly reduced those of IL-6. These data suggest that Epo might act as a protective cytokine in inflammatory pathologies of the CNS [1].

5.4. Spinal cord injury models

Epo significantly improves functional outcome in compression and contusion models of spinal cord injury in rats [39]. In the contusion model of more severe spinal cord injury, secondary inflammation was also markedly attenuated by Epo administration [39] and protection has been confirmed by electron microscopy [50]. Asialoerythropoietin (asialoEpo), generated by total enzymatic desialylation of rhEpo, has beneficial effect in the compression model of experimental spinal cord injury [34].

5.4.1. Peripheral nerve injury models

In a unilateral sciatic nerve transection model in neonatal rats, systemically administered Epo for 2 weeks significantly prevented the loss of motor neurons [42]. A single dose of asialoEpo significantly decreased functional loss in the sciatic nerve crush model in adult rats [34]. These findings suggest the potential use of Epo in treating diseases that involve degeneration and death of motor neurons, such as motor neuropathy and amyotrophic lateral sclerosis.

5.4.2. Retinal ischemia models

In a model of transient global retinal ischemia induced by raising intraocular pressure, which is a clinically relevant model for retinal diseases, systemic administration of Epo before or immediately after retinal ischemia reduces histopathological damage and apoptotic cell death and promotes functional recovery as assessed by electroretinography [45]. Neutralization of endogenous Epo with soluble EpoR exacerbates retinal ischemic injury, suggesting a crucial role for an endogenous Epo/EpoR system in the survival and recovery of neurons after an ischemic insult. Systemically applied Epo crosses the blood retina barrier and prevents apoptosis even when given therapeutically after light insult of adult mouse retina [41]. Thus, Epo may, through the inhibition of apoptosis, be beneficial for the treatment of different forms of retinal disease.

6. Mechanisms of erythropoietin neuroprotection

Questions about the mechanisms by which Epo is neuroprotective have not been fully answered. Epo may act in a coordinated fashion at multiple levels, including limiting the production of tissue-injuring molecules such as reactive oxygen species and glutamate, reversal of vasospasm, stimulation of angiogenesis, attenuation of apoptosis, modulation of inflammation and recruitment of stem cells. Thus, Epo may protect neurons by a combination of these mechanisms.

The EpoR belongs to the cytokine receptor superfamily for which substantial information concerning signaling biology exists [107]. Therefore, not only is it possible that Epo may have some of the same functions as in hematopoiesis, it may also use some of the same signaling pathways. When Epo binds EpoR, it causes dimerization of the receptor, autophosphorylation of Janus-tyrosine-kinase-2 (JAK-2) and receptor activation (Fig. 1).

JAK-2 activation leads to several downstream signaling pathways including Ras-mitogen activated protein kinase (MAPK), phosphatidylinositol-3-kinase [PI(3)K] and the transcription factor signal transducers and activators of transcription 5 (STAT-5) (Fig. 1). The net effect of EpoR stimulation in the target cell is proliferation, inhibition of apoptosis and differentiation [107]. Receptor inactivation is mediated by SHP-1 dephosphorylation [44].

Distinct intracellular signaling cascades that have been characterized in hematopoietic cell lines are functional in neurons [90]. Even more importantly, these pathways are crucial for the neuroprotective effect of Epo since specific inhibitors of MAPK and PI(3)K pathways largely abolish the Epo-induced protection against hypoxia-induced cell death [90]. Epo acts through the EpoR, enhancing the phosphorylation of the MAPK and inducing tyrosine phosphorylation of JAK-2 [15,16]. These signaling mechanisms have been shown to be involved in the cytoprotective effects of other growth factors such as that of brain-derived neurotrophic factor and vascular endothelial growth factor (VEGF) [89]. Interaction between the JAK-2, STAT-5 and the nuclear factor-κB (NFκB) signal transduction systems in neurons has recently been demonstrated [31]. Epo stimulation produces a dose-dependent increase in nuclear NFκB and concomitant decrease in cytoplasmic NFκB, and these effects were required for neuroprotection. JAK-2 phosphorylation was also required for nuclear translocation of NFκB and for Epo-mediated neuroprotection in this system [31]. However, these interactions between signaling pathways have not been

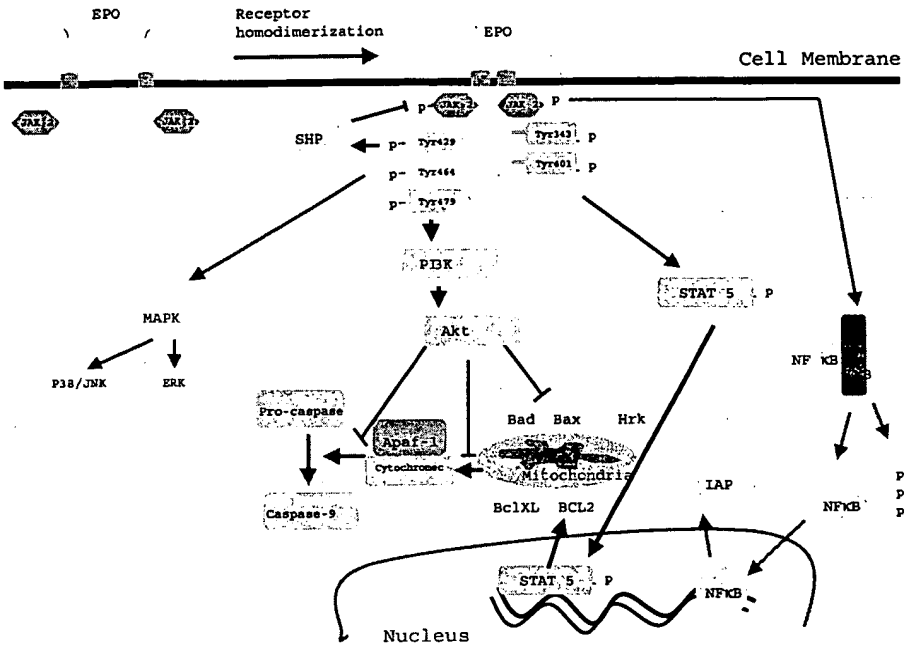


Fig. 1. A schematic illustration of intracellular signaling events resulting from Epo binding to the EpoR in neuronal cells is shown. Epo binding to its receptor activates intracellular signaling through a conformational change that leads to phosphorylation of JAK-2. Phosphorylation of JAK-2 mediates phosphorylation of STAT-5, PI(3)K, MAPK and inhibitor of the transcription factor NFκB (IκB). NFκB dissociates from IκB. STAT-5 and NFκB translocate to the nucleus and bind to DNA, promoting and expression of neuroprotective genes such as bcl-XL and bcl-2. EpoR inactivation is mediated by SHP-1 dephosphorylation. Epo: Erythropoietin; EpoR: erythropoietin receptor; JAK-2: Janus-tyrosine kinase-2; STAT-5: signal transducer and activator of transcription-5; NFκB: nuclear factor-κB; MAPK: mitogen-activated protein kinase; PI(3)K: phosphatidylinositol-3-kinase; IAP: inhibitor of apoptotic protein.

demonstrated in erythroid cell lines [24]. In addition, signaling events addressed in the context of Epo mechanisms exhibit complexity in different systems and have dual roles in cellular processes such as apoptosis [24,31].

Epo regulates a variety of neural cell functions, including calcium flux, membrane depolarization and neurotransmitter synthesis, as well as cell survival. Epo increases synaptic transmission in hippocampal slice cultures, and this effect is blocked by a JAK2 inhibitor [100]. This finding suggests that Epo improves synaptic transmission during and following ischemia in hippocampal slice cultures. Epo may be involved in synaptic plasticity via the inhibition or stimulation of various neurotransmitters. Epo stimulates dopamine release and tyrosine hydroxylase activity in PC12 cells, through the activation of calcium channels, induces membrane depolarization, stimulates MAPK activity and increases NO synthesis [54]. Since NO is reported to stimulate the release of different neurotransmitters, such as γ-aminobutyric acid, dopamine and acetylcholine, it has been suggested that Epo may also partly stimulate neurotransmitter release by inducing NO production. However, Epo stimulates the release of dopamine and acetylcholine from hippocampal and striatal slices in rats, but this occurs independent of NO production [105]. Epo and Epomimetic peptide 1, an EpoR agonist, inhibit the calcium-induced release of dopamine from PC12 cells, by activating JAK2, which is known to be linked with EpoR [51]. These discrepancies between studies may result from the use of different culture systems. Similar to signaling events mentioned above, NO exhibits dual properties in physiologic and pathological conditions in the nervous system. NO is a transmitter and immunomodulator and is implicated in an enormous number of biological functions both in physiological and pathological conditions. Often, it is not clear if it plays a deleterious or beneficial role. There is still a demand for a detailed analysis of the effect of Epo on NO production from different cellular sources and the expression of different isoforms of NOS both in physiological and pathological conditions.

Epo reduces calcium-induced glutamate release from cultured cerebellar granule neurons [52]. EpoR activation protects hippocampal neurons from ischemic neuronal damage through the inhibition of glutamate release via exocytosis in hippocampal slice cultures. Activation of EpoR attenuates calcium-induced glutamate release from cerebellar granule cells and hippocampal neurons possibly through activation of JAK2 tyrosine kinase. It has been reported that exposure to Epo for at least 8 h before the excitotoxic insult is required for protection from glutamate toxicity, and protection is dependent on RNA and protein synthesis [70]. However, Epo confers protection very quickly by inhibiting presynaptic functions [52]. Thus, the activation of EpoR appears to protect neurons from ischemic damage through at least two distinct mechanisms, and presynaptic and postsynaptic mechanisms are likely to be involved in inducing protection in the early and late phases of ischemia, respectively. Recently, it has been demonstrated that Epo

stimulates the activity of T-type voltage-dependent calcium channels [5]. Patch-clamp studies of the human neuroblastoma cell line SK-N-MC cells confirmed the expression of T-type Ca^{2+} channels, whose peak macroscopic current was increased by the addition of Epo to the bathing medium, and confocal laser scanning microscopy analysis of these cells confirmed a transient increase in intracellular free [Ca^{2+}] in response to externally applied rhEpo [5]. Thus, Epo can interact with neuronal cells by affecting Ca^{2+} homeostasis through an increase in Ca^{2+} influx via plasma membrane T-type voltage-dependent Ca^{2+} channels. The EpoR domain required for calcium channel activation has been identified [67]. It has been suggested that many functions of Epo in the nervous system may be mediated by activation of calcium channels [56,70]. The addition of Epo to PC12 neuronal cells results in an increase of intracellular Ca^{2+} and monoamine concentrations [64].

Another mechanism that may contribute to the cytoprotective potential of Epo is antioxidation. It has been proposed that suppression of formation of NO-mediated free radicals or antagonism of their toxicity underlies the neuroprotective effect of Epo [83]. Epo may exert its neuroprotective action by reducing the NO-mediated formation of free radicals or by antagonizing their toxicity [14,54]. Epo may increase the activity of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase, in neurons, thus protecting the brain parenchyma from ischemic damage. An antioxidant action of Epo is further supported by the findings that Epo protects against oxidative damage via inhibition of lipid peroxidation and by restoration of cytosolic catalase and glutathione peroxidase activities in erythrocytes [21]. Systemic posttreatment with Epo reduced post-ischemic lipid peroxidation in a global cerebral ischemia model of gerbils, ischemia-perfusion-induced fetal oxidative damage and experimental spinal cord injury [14,50,92]. Epo increases glutathione peroxidase activity in cultured mouse astrocytes [38].

Evidence for an intrinsic anti-apoptotic brain Epo release is provided by the findings that intracerebral infusion of soluble EpoR to neutralize endogenously produced Epo resulted in massive CA1 neuronal apoptosis after mild nonlethal global cerebral ischemia [83].

Exogenous Epo may reduce the brain ischemia area by protecting endothelial and neural cells from apoptotic cell death [7]. Recent studies confirm the anti-apoptotic effect of Epo in a variety of in vitro and in vivo neuronal injury models [19,39,41,45,48,56,90]. In cultured rat motoneurons, Epo inhibited apoptosis induced by serum deprivation or kainic acid exposure [90]. Protection in this system required pretreatment with Epo, consistent with the induction of a gene expression program [90]. Activation of neuronal EpoR prevents apoptosis induced by *N*-methyl-D-aspartate (NMDA) or NO by triggering cross talk between JAK-2 and NFκB [31]. Epo also prevents spontaneous apoptosis in rodent microglia cultures [97]. Epo may exert its anti-apoptotic effect via the differential regulation of the expression of genes involved in the apoptotic process [24]. Epo modulates a series of death-related cellular pathways during anoxia and free radical induced neuronal injury in vitro [28]. Through pathways that involve the initial activation of protein kinase B, Epo maintains mitochondrial membrane potential. Subsequently, Epo inhibits caspase 8-, caspase 1-, and caspase 3-like activities linked to cytochrome *c* release [25,28]. In free-radical injury model, Epo modulates neuronal apoptotic membrane phosphatidylserine (PS) expression, increases Akt1 activity, phosphorylates pro-apoptotic Bad protein and maintains neuronal nuclear DNA integrity through the downstream modulation of mitochondrial membrane potential, cytochrome *c* release and caspase 1-, 3- and 8-like activities [25]. A microarray study showed that Epo causes changes in the expression of genes involved in cell proliferation, cell differentiation and cell survival in differentiated PC12 cells [78]. A number of genes that have been previously implicated in Epo signaling in hematopoietic cells were also found to be regulated by Epo in neuronal cells. Epo consistently increases the expression of the anti-apoptotic gene bcl-XL and decreases the expression of pro-apoptotic gene bak in PC12 cells. These findings suggest that the neuroprotective effects of Epo may involve controlling the balance of expression between pro- and anti-apoptotic molecules [78]. Epo is able to shift the Bcl/Bax ratio towards a net anti-apoptotic effect in cultured microglia [97]. It also up-regulates bcl-XL mRNA and protein expressions in cultured neurons [101]. Epo rescued hippocampal CA1 neurons from lethal ischemic damage and up-regulated the expressions of bcl-XL mRNA and protein in the hippocampal CA1 field of ischemic gerbils. Thus, Epo prevents delayed neuronal death of hippocampal neurons, possibly through up-regulation of bcl-XL, which is known to facilitate neuronal survival [101].

Stimulation of neovascularization is another potentially protective mechanism activated by Epo that may help preserve perfusion in metabolically compromised tissue as brain endothelial cells express EpoR and respond to Epo treatment with proliferation [62,104]. Moreover, angiogenesis after Epo treatment has been reported in vitro and in vivo [13,22,79,106]. Epo and EpoR expression are up-regulated by vascular endothelium, as well as by neurons and glial cells after infarction in the human brain, suggesting a role for Epo and its receptor in the vascular response to brain injury [91]. Epo may modulate angiogenesis in the ischemic brain, thus improving the blood flow and tissue oxygenation in the border zone of the ischemic area. [62]. Functional EpoR is present on endothelial cells [4,17, 71,104] and stimulation of endothelial cells by rhEpo induces a proangiogenic phenotype which includes the production of proteases (which enhance the ability of cells to migrate), increased cell migration and cell proliferation followed by redifferentiation [80]. Epo also interacts synergistically with VEGF, a potent angiogenic factor, to potentiate its vascular activity, and these two growth factors, both

of which are regulated by HIF-1, often co-distribute [3]. Epo prevents anoxia-induced vascular injury in endothelial cells [27]. Protection by Epo is dependent on the activation of protein kinase B (Akt1) and the maintenance of mitochondrial membrane potential. Epo inhibits caspase 8-, caspase 1- and caspase 3-like activities that were linked to mitochondrial cytochrome *c* release in this model [26].

The actions of Epo are not limited to directly influencing cell survival, as Epo is trophic in cultured neuronal cells [16,53,90,96]. These findings suggest that inhibition of neuronal apoptosis underlies short latency protective effects of Epo following cerebral ischemia and other brain injuries. The neurotrophic actions implicate longer-latency effects as well. Reactive astrocytes in older ischemic lesions express Epo immunoreactivity emphasizing the regenerative and secondarily preventive potential of Epo in addition to its early neuroprotective effects [89,91].

Another possible mechanism that is responsible for the long-latency effects may be modulation of neurogenesis. Epo may function in the control of proliferation and differentiation of neuronal stem cells. Its receptors are expressed in the embryonic germinal zone during neurogenesis as well as in the adult subventricular zone, which continues to generate neurons throughout adulthood [87]. Cultured neural stem cells under hypoxia produce two- to threefold more neurons and this is associated with an elevation in Epo mRNA expression. Epo infusion into the adult rat lateral ventricles results in a decrease in the numbers of neural stem cells in the subventricular zone, an increase in newly generated cells migrating to the olfactory bulb and an increase in new olfactory bulb interneurons. These findings suggest that Epo is capable of regulating the production of neuronal progenitor cells by forebrain neural stem cells [87]. It has also been demonstrated that Epo is a mediator for dopaminergic neuron differentiation from CNS precursors under low oxygen conditions [94]. Epo mediates ascorbate-induced dopaminergic differentiation from embryonic mesencephalic precursors [57].

An anti-inflammatory effect has also been attributed to Epo in the light of in vivo studies [1,11,19,39]. EpoR has been demonstrated in polymorphonuclear leukocytes [86] and Epo decreases pro-inflammatory cytokine production and inflammation in cerebral ischemia and EAE [1,98]. However, Epo does not affect the pro-inflammatory function of microglial cells in vitro [97]. Epo modulates neuronal apoptotic membrane PS exposure necessary for microglial activation primarily through the regulation of caspase 1 [25]. Thus, Epo offers extrinsic protection through the inhibition of cerebral microglial activation and the suppression of microglial PS receptor expression for the prevention of neuronal phagocytosis.

When a stimulus that is capable of causing injury to a tissue is applied close to the threshold of damage, endogenous protective mechanisms are activated, thus potentially lessening the impact of subsequent, more severe stimuli. For example, a subthreshold ischemic or hypoxic insult applied to the brain activates certain cellular pathways that reduce the damage caused by subsequent ischemic episodes. This phenomenon is referred to as ischemic and hypoxic preconditioning or tolerance and may provide an opportunity to use these endogenous mechanisms in the clinic to treat patients with stroke and other CNS disorders [32,36]. Recent in vitro and in vivo studies suggest that Epo is an essential mediator of ischemic and hypoxic preconditioning. Hypoxia elicits a delayed, short-lasting tolerance to focal permanent ischemia in the adult mouse brain, and HIF-1 target genes (Epo and VEGF) could contribute to the establishment of tolerance [9,77]. Signaling cascades that mediate ischemic–hypoxic preconditioning have been proposed from a recent in vitro study of an oxygen–glucose deprivation model [81]. After HIF-1 activation by hypoxia in the astrocytes, these cells express and release Epo. Epo activates the neuronal Epo receptor and, subsequently, JAK-2 and PI(3)K. PI(3)K deactivates pro-apoptotic Bad protein via Akt-mediated phosphorylation and so may inhibit hypoxia-induced apoptosis in neurons. Thus, Epo synthesized by hypoxic astrocytes may mediate the protective phenomenon of preconditioning in the nervous system in which exposure to a brief, nontoxic episode of ischemia increases the resistance of neurons to subsequent more severe insults [81].

Although current literature cited in this section provides some implications on the mechanisms of Epo neuroprotection, further studies are needed to reveal exact causal relationship between these mechanisms and Epo neuroprotection.

7. Use of erythropoietin as a neurotherapeutic agent in humans

Evidence from cell culture and animal experiments systematically reviewed above indicates a neuroprotective function for Epo. Neuroprotection as a tool to prevent or oppose neuronal loss in CNS disorders with various pathophysiological origins, such as cerebral ischemia, hypoxia, trauma and inflammatory and neurodegenerative diseases, represents a novel therapeutic approach. This approach is supported by a large body of experimental evidence on cell culture and experimental animal studies demonstrating beneficial effects of growth factors on neuronal survival and functional recovery. However, the clinical use of neuroprotective agents has been hampered by the toxicity of many of the growth factors that have proved to be efficient neuroprotective agents in animal studies [89]. Thus, safe and well-tolerated neuroprotective agents are still wanted in the clinical neuroscience area. rhEpo is an extremely well-tolerated compound, used in millions of patients [43]. This strongly supports evaluation of Epo for neuroprotective therapies in the clinical setting. The therapeutic potential of Epo ranges from acute and chronic neurodegenerative diseases (stroke, Parkinson's disease, Alzheimer disease, amyo-

trophic lateral sclerosis, multiple sclerosis, neurotrauma and perinatal asphyxia) to psychiatric disorders such as schizophrenia, where neurodegenerative processes are likely to contribute to the pathophysiology of the disease [89]. A recent double-blind randomized proof-of-concept clinical trial has demonstrated significant improvement in outcome of stroke patients who were administered rhEpo intravenously within 8 h of the onset of symptoms [33]. No safety concerns were identified in this study. In addition, a phase I trial, funded by National Institutes of Health, using high-dose rhEpo to treat asphyxiated newborns has begun [47].

A potential problem in the chronic use of Epo for neurodegenerative disorders will be the undesirable erythropoietic side effects [102]. The need for nonerythropoietic rhEpo derivatives still retaining neuroprotective action has led to the discovery of asialoEpo, generated by total enzymatic desialylation of rhEpo [34]. The strategy to develop derivatives of rhEpo lacking erythropoietic activity, but retaining neuroprotective potential, may allow for multiple and chronic usage of Epo in neurodegenerative disorders.

In conclusion, recent studies in the past decade suggest that Epo is a potential novel neurotherapeutic agent and further clinical studies are warranted.

References

[1] D. Agnello, P. Bigini, P. Villa, T. Menini, A. Cerami, M.L. Brines, P. Ghezzi, Erythropoietin exerts anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis, Brain Res. 952 (2002) 128–134.

[2] C. Alafaci, F. Salpietro, G. Grasso, A. Sfacteria, M. Passalacqua, A. Morabito, E. Tripodo, G. Calapai, M. Buemi, F. Tomasello, Effect of recombinant human erythropoietin on cerebral ischemia following experimental subarachnoid hemorrhage, Eur. J. Pharmacol. 406 (2000) 219–225.

[3] M.V. Alvarez Arroyo, M.A. Castilla, F.R. Gonzalez Pacheco, D. Tan, A. Riesco, S. Casado, C. Caramelo, Role of vascular endothelial growth factor on erythropoietin-related endothelial cell proliferation, J. Am. Soc. Nephrol. 9 (1998) 1998–2004.

[4] A. Anagnostou, Z. Liu, M. Steiner, K. Chin, E.S. Lee, N. Kessimian, C.T. Noguchi, Erythropoietin receptor mRNA expression in human endothelial cells, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 3974–3978.

[5] R. Assandri, M. Egger, M. Gassmann, E. Niggli, C. Bauer, I. Foster, A. Gorlach, Erythropoietin modulates intracellular calcium in a human neuroblastoma cell line, J. Physiol. 516 (1999) 343–352.

[6] D. Banerjee, M. Rodriguez, M. Nag, J.W. Adamson, Exposure of endothelial cells to recombinant human erythropoietin induces nitric oxide synthase activity, Kidney Int. 57 (2000) 1895–1904.

[7] M. Bernaudin, H.H. Marti, S. Roussel, D. Divoux, A. Nouvelot, E.T. MacKenzie, E. Petit, A potential role for erythropoietin in focal permanent cerebral ischemia in mice, J. Cereb. Blood Flow Metab. 19 (1999) 643–651.

[8] M. Bernaudin, A. Bellail, H.H. Marti, A. Yvon, D. Vivien, I. Duchatelle, E.T. Mackenzie, E. Petit, Neurons and astrocytes express Epo mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain, Glia 30 (2000) 271–278.

[9] M. Bernaudin, A.-S. Nedelec, D. Divoux, E.T. MacKenzie, E. Petit, P. Schumann-Bard, Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxia-inducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain, J. Cereb. Blood Flow Metab. 22 (2002) 393–403.

[10] M. Brines, What evidence supports use of erythropoietin as a novel neurotherapeutic, Oncology 16 (2002) 79–89.

[11] M.L. Brines, P. Ghezzi, S. Keenan, D. Agnello, N.C. de Lanerolle, C. Cerami, L.M. Itri, A. Cerami, Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 10526–10531.

[12] M. Buemi, A. Allegra, C. Aloisi, F. Corica, N. Frisina, Hemodynamic effects of recombinant human erythropoietin, Nephron 81 (1999) 1–4.

[13] M. Buemi, M. Vaccaro, A. Sturiale, M.R. Galeano, C. Sansotta, V. Cavallari, F. Floccari, D. D'Amico, V. Torre, G. Calapai, N. Frisina, F. Guarneri, G. Vermiglio, Recombinant human erythropoietin influences revascularization and healing in a random model of ischemic flaps in rat, Acta Derm. Venereol. 82 (2002) 411–417.

[14] G. Calapai, M.C. Marciano, F. Corica, A. Allegra, A. Parisi, N. Frisina, A.P. Caputi, M. Buemi, Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation, Eur. J. Pharmacol. 401 (2000) 349–356.

[15] W.M. Campana, R.R. Myers, Erythropoietin and erythropoietin receptors in the peripheral nervous system: changes after nerve injury, FASEB J. 15 (2001) 1804–1806.

[16] W.M. Campana, R. Misasi, J.S. O'Brien, Identification of a neurotrophic sequence in erythropoietin, Int. J. Mol. Med. 1 (1998) 235–241.

[17] R.G. Carlini, E.J. Alonzo, J. Dominguez, I. Blanca, J.R. Weisinger, M. Rothstein, E. Bellorin-Font, Effect of recombinant human erythropoietin on endothelial cell apoptosis, Kidney Int. 55 (1999) 546–553.

[18] M.A. Catania, M.C. Marciano, A. Parisi, A. Sturiale, M. Buemi, G. Grasso, F. Squadrito, A.P. Caputi, G. Calapai, Erythropoietin prevents cognition impairment induced by transient brain ischemia in gerbils, Eur. J. Pharmacol. 437 (2002) 147–150.

[19] M. Celik, N. Gokmen, S. Erbayraktar, M. Akhisaroglu, S. Konakci, C. Ulukus, S. Genc, K. Genc, E. Sagiroglu, A. Cerami, M. Brines, Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 2258–2263.

[20] N.S. Chandel, E. Maltepe, E. Goldwasser, C.E. Mathieu, M.C. Simon, P.T. Schumacker, Mitochondrial reactive oxygen species trigger hypoxia-induced transcription, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 11715–11720.

[21] A. Chattopadhyay, T.D. Choudhury, D. Bandyopadhyay, A.G. Datta, Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical, Biochem. Pharmacol. 59 (2000) 419–425.

[22] M. Chikuma, S. Masuda, T. Kobayashi, M. Nagao, R. Sasaki, Tissue-specific regulation of erythropoietin production in the murine kidney, brain and uterus, Am. J. Physiol. Endocrinol. Metab. 279 (2000) E1242–E1248.

[23] K. Chin, X. Yu, B. Beleslin-Cokic, C. Liu, K. Shen, H.W. Mohrenweiser, C.T. Noguchi, Production and processing of erythropoietin receptor transcripts in brain, Mol. Brain Res. 81 (2000) 29–42.

[24] Z.Z. Chong, J.-Q. Kang, K. Maiese, Hematopoietic factor erythropoietin fosters neuroprotection through novel signal transduction cascades, J. Cereb. Blood Flow Metab. 22 (2002) 503–514.

[25] Z.Z. Chong, J.Q. Kang, K. Maiese, Erythropoietin fosters both intrinsic and extrinsic neuronal protection through modulation of microglia, Akt1, Bad, and caspase-mediated pathways, Br. J. Pharmacol. 138 (2003) 1107–1118.

[26] Z.Z. Chong, J.Q. Kang, K. Maiese, Apaf-1, Bcl-x_L, cytochrome, and caspase-9 form the critical elements for cerebral vascular protection by erythropoietin, J. Cereb. Blood Flow Metab. 23 (2003) 320–330.

[27] Z.Z. Chong, J.Q. Kang, K. Maiese, Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases, Circulation 106 (2002) 2973–2979.
[28] Z.Z. Chong, S.H. Lin, J.Q. Kang, K. Maiese, Erythropoietin prevents early and late neuronal demise through modulation of Akt1 and induction of caspase 1, 3, and 8, J. Neurosci. Res. 71 (2003) 659–669.
[29] C. Dame, P. Bartmann, E.M. Wolber, H. Fahnenstich, D. Hofmann, J. Fandrey, Erythropoietin gene expression in different areas of the developing human central nervous system, Dev. Brain Res. 125 (2000) 69–74.
[30] M. Digicaylioglu, S. Bichet, H.H. Marti, R.H. Wenger, L.A. Rivas, C. Bauer, M. Gassman, Localization of specific erythropoietin binding sites in defined areas of the mouse brain, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 3717–3720.
[31] M. Digicaylioglu, S.A. Lipton, Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades, Nature 412 (2001) 641–647.
[32] U. Dirnagl, R.P. Simon, J.M. Hallenbeck, Ischemic tolerance and endogenous neuroprotection, Trends Neurosci. 26 (2003) 248–254.
[33] H. Ehrenreich, M. Hasselblatt, C. Dembowski, L. Cepek, P. Lewczuk, M. Stiefel, H.H. Rustenbeck, N. Breiter, S. Jacob, F. Knerlich, M. Bohn, W. Poser, E. Ruther, M. Kochen, O. Gefeller, C. Gleiter, T.C. Wessel, M. De Ryck, L. Itri, H. Prange, A. Cerami, M. Brines, A.L. Siren, Erythropoietin therapy for acute stroke is both safe and beneficial, Mol. Med. 8 (2002) 495–505.
[34] S. Erbayraktar, G. Grasso, A. Sfacteria, Q.W. Xie, T. Coleman, M. Kreilgaard, L. Torup, T. Sager, Z. Erbayraktar, N. Gokmen, O. Yilmaz, P. Ghezzi, P. Villa, M. Fratelli, S. Casagrande, M. Leist, L. Helboe, J. Gerwein, S. Christensen, M.A. Geist, L.O. Pedersen, C. Cerami-Hand, J.P. Wuerth, A. Cerami, M. Brines, Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 6741–6746.
[35] E.S. Fenjves, M.S. Ochoa, O. Cabrera, A.J. Mendez, N.S. Kenyon, L. Inverardi, C. Ricordi, Human, nonhuman primate, and rat pancreatic islets express erythropoietin receptors, Transplantation 75 (2003) 1356–1360.
[36] M. Fisher, R. Ratan, New perspectives on developing acute stroke therapy, Ann. Neurol. 53 (2003) 10–20.
[37] S. Genc, F. Kuralay, K. Genc, M. Akhisaroglu, S. Fadiloglu, K. Yorukoglu, M. Fadiloglu, A. Gure, Erythropoietin exerts neuroprotection in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated C57/BL mice via increasing nitric oxide production, Neurosci. Lett. 298 (2001) 139–141.
[38] S. Genc, M. Akhisaroglu, F. Kuralay, K. Genc, Erythropoietin restores glutathione peroxidase activity in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity in C57BL mice and stimulates murine astroglial glutathione peroxidase production in vitro, Neurosci. Lett. 321 (2002) 73–76.
[39] A. Gorio, N. Gokmen, S. Erbayraktar, O. Yılmaz, L. Madaschi, C. Cichetti, A.M. Di Giulio, E. Vardar, A. Cerami, M. Brines, Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 9450–9455.
[40] G. Grasso, M. Buemi, C. Alafaci, A. Sfacteria, M. Passalacqua, A. Sturiale, G. Calapai, G. De Vico, G. Piedimonte, F.M. Salpietro, F. Tomasello, Beneficial effects of systemic administration of recombinant human erythropoietin in rabbits subjected to subarachnoid hemorrhage, Proc. Natl. Acad. Sci. U. S. A. 99 2002, pp. 5627–5631.
[41] C. Grimm, A. Wenzel, M. Groszer, H. Mayser, M. Seeliger, M. Samardzija, C. Bauer, M. Gassmann, C.E. Reme , HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration, Nat. Med. 8 (2002) 718–724.
[42] Y. Iwasaki, K. Ikeda, Y. Ichikawa, O. Igarashi, K. Iwamoto, M. Kinoshita, Protective effect of interleukin-3 and erythropoietin on motor neuron death after neonatal axotomy, Neurol. Res. 24 (2002) 643–646.
[43] W. Jelkmann, Erythropoietin: structure, control of production, and function, Physiol. Rev. 72 (1992) 449–489.
[44] H. Jiao, K. Berrada, W. Yang, M. Tabrizi, L.C. Platanias, T. Yi, Direct association with and dephosphorylation of JAK2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1, Mol. Cell Biol. 16 (1996) 6985–6992.
[45] A.K. Junk, A. Mammis, S.I. Savitz, M. Singh, S. Roth, S. Malhotra, P.S. Rosenbaum, A. Cerami, M. Brines, D.M. Rosenbaum, Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 10659–10664.
[46] S.E. Juul, A.E. Joyce, Y. Zhao, D.J. Ledbetter, Why is erythropoietin present in human milk? Studies of erythropoietin receptors on enterocytes of human and rat neonates, Pediatr. Res. 46 (1999) 263–268.
[47] S. Juul, Erythropoietin in the central nervous system, and its use to prevent hypoxic–ischemic brain damage, Acta Paediatr. 438 (2002) 36–42.
[48] S.E. Juul, D.K. Anderson, Y. Li, R.D. Christensen, Erythropoietin and erythropoietin receptor in the developing human central nervous system, Pediatr. Res. 43 (1998) 40–49.
[49] S.E. Juul, A. Yachnis, A.M. Rojiani, R.D. Christensen, Immunohistochemical localization of erythropoietin and its receptor in the developing human brain, Pediatr. Dev. Pathol. 2 (1999) 148–158.
[50] E. Kaptanoglu, I. Solaroglu, O. Okutan, H.S. Surucu, F. Akbiyik, E. Beskonakli, Erythropoietin exerts neuroprotection after acute spinal cord injury in rats: effect on lipid peroxidation and early ultrastructural findings, Neurosurg. Rev. (2003) Aug. 14 [Epub ahead of print].
[51] M. Kawakami, S. Iwasaki, K. Sato, M. Takahashi, Erythropoietin inhibits calcium-induced neurotransmitter release from clonal neuronal cells, Biochem. Biophys. Res. Commun. 279 (2000) 293–297.
[52] M. Kawakami, M. Sekiguchi, K. Sato, S. Kozaki, M. Takahashi, Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia, J. Biol. Chem. 276 (2001) 39469–39475.
[53] Y. Konishi, D.H. Chui, H. Hirose, T. Kunishita, T. Tabira, Trophic effect of erythropoietin and other hematopoietic factors on central cholinergic neurons in vitro and in vivo, Brain Res. 609 (1993) 29–35.
[54] K. Koshimura, Y. Murakami, M. Sohmiya, J. Tanaka, Y. Kato, Effects of erythropoietin on neuronal activity, J. Neurochem. 72 (1999) 2565–2572.
[55] A. Kumral, E. Ozer, O. Yilmaz, M. Akhisaroglu, N. Gokmen, N. Duman, C. Ulukus, S. Genc, H. Ozkan, Neuroprotective effect of erythropoietin on hypoxic–ischemic brain injury in neonatal rats, Biol. Neonate 83 (2003) 224–228.
[56] A. Kumral, H. Baskin, N. Gokmen, O. Yılmaz, K. Genc, S. Genc, H. Ozkan, Selective inhibition of nitric oxide in hypoxic–ischemic brain model in newborn rats: is it an explanation for the protective role of erythropoietin? Biol. Neonate 85 (2004) 51–54.
[57] J.Y. Lee, H.C. Koh, M.Y. Chang, C.H. Park, Y.S. Lee, S.H. Lee, Erythropoietin and bone morphogenic protein 7 mediate ascorbate-induced dopaminergic differentiation from embryonic mesencephalic precursors, NeuroReport 14 (2003) 1401–1404.
[58] P. Lewczuk, M. Hasselblatt, H. Kamrowski-Kruck, A. Heyer, C. Unzicker, A.L. Siren, H. Ehrenreich, Survival of hippocampal neurons in culture upon hypoxia: effect of erythropoietin, NeuroReport 11 (2000) 3485–3488.
[59] Y. Li, S.E. Juul, J.A. Morris-Wiman, D.A. Calhoun, R.D. Christensen, Erythropoietin receptors are expressed in the central nervous system of mid-trimester human fetuses, Pediatr. Res. 40 (1996) 376–380.
[60] C. Liu, K. Shen, Z. Liu, C.T. Noguchi, Regulated human erythropoietin receptor expression in mouse brain, J. Biol. Chem. 272 (1997) 32395–32400.
[61] Z.Y. Liu, K. Chin, C.T. Noguchi, Tissue specific expression of hu-

man erythropoietin receptor in transgenic mice, Dev. Biol. 166 (1994) 159–169.
[62] H.H. Marti, M. Bernaudin, E. Petit, C. Bauer, Neuroprotection and angiogenesis: dual role of erythropoietin in brain ischemia, News Physiol. Sci. 15 (2000) 225–229.
[63] H.H. Marti, R.H. Wenger, L.A. Rivas, U. Straumann, M. Digicaylioglu, V. Henn, Y. Yonekawa, C. Bauer, M. Gassmann, Erythropoietin gene expression in human, monkey and human brain, Eur. J. Neurosci. 8 (1996) 666–676.
[64] S. Masuda, M. Nagao, K. Takahata, Y. Konishi, F. Gallyas, T. Tabira, R. Sasaki, Functional erythropoietin receptor of the cells with neural characteristics: comparison with receptor properties of erythroid cells, J. Biol. Chem. 268 (1993) 11208–11216.
[65] S. Masuda, M. Okano, K. Yamagishi, M. Nagao, M. Ueda, R. Sasaki, A novel site of erythropoietin production: oxygen-dependent production in cultured rat astrocytes, J. Biol. Chem. 269 (1994) 19488–19493.
[66] S. Masuda, M. Chikuma, R. Sasaki, Insulin-like growth factors and insulin stimulate erythropoietin production in primary cultured astrocytes, Brain Res. 746 (1997) 63–70.
[67] B.A. Miller, D.L. Barber, L.L. Bell, B.K. Beattie, M.Y. Zhang, B.G. Neel, M. Yoakim, L.I. Rothblum, J.Y. Cheung, Identification of the erythropoietin receptor domain required for calcium channel activation, J. Biol. Chem. 274 (1999) 20465–20472.
[68] N. Morakkabati, F. Gollnick, R. Meyer, J. Fandrey, W. Jelkmann, Erythropoietin induces Ca^{2+} mobilization and contraction in rat mesangial and aortic smooth muscle cultures, Exp. Hematol. 24 (1996) 392–397.
[69] E. Morishita, H. Narita, M. Nishida, N. Kawashima, K. Yamagishi, S. Masuda, M. Nagao, H. Hatta, R. Sasaki, Anti-erythropoietin receptor monoclonal antibody: epitope mapping, quantification of the soluble receptor, and detection of the solubilized transmembrane receptor and the receptor-expressing cells, Blood 88 (1996) 465–471.
[70] E. Morishita, S. Masuda, M. Nagao, Y. Yasuda, R. Sasaki, Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death, Neuroscience 76 (1997) 105–116.
[71] T. Nagai, T. Akizawa, Y. Nakashima, S. Kohjiro, K. Nabeshima, N. Kanamori, K. Takayama, E. Kinugasa, S. Koshikawa, Effects of rhuEpo on cellular proliferation and endothelin-1 production in cultured endothelial cells, Nephrol., Dial., Transplant 10 (1995) 1814–1819.
[72] A. Nagai, E. Nakagawa, H.B. Choi, K. Hatori, S. Kobayashi, S.U. Kim, Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia, and oligodendrocytes grown in culture, J. Neuropathol. Exp. Neurol. 60 (2001) 386–392.
[73] A.R. Nissenson, Epoetin and cognitive function, Am. J. Kidney Dis. 20 (1992) 21–24.
[74] M. Ogilvie, X. Yu, V. Nicolas-Metral, S.M. Pulido, C. Liu, U.T. Ruegg, C.T. Noguchi, Erythropoietin stimulates proliferation and interferes with differentiation of myoblasts, J. Biol. Chem. 275 (2000) 39754–39761.
[75] A. Okada, Y. Kinoshita, T. Maekawa, M.S. Hassan, C. Kawanami, M. Asahara, Y. Matsushima, K. Kishi, H. Nakata, Y. Naribayashi, T. Chiba, Erythropoietin stimulates proliferation of rat-cultured gastric mucosal cells, Digestion 57 (1996) 328–332.
[76] J.L. Pickett, D.C. Theberege, W.S. Brown, S.U. Schweitzer, A.R. Nissenson, Normalizing hematocrit in dialysis patients improves brain function, Am. J. Kidney Dis. 33 (1999) 1122–1130.
[77] K. Prass, A. Scharff, K. Ruscher, D. Lowl, C. Muselmann, I. Victorov, K. Kapinya, U. Dirnagl, A. Meisel, Hypoxia-induced stroke tolerance in the mouse is mediated by erythropoietin, Stroke 34 (2003) 1981–1986.
[78] M.J. Renzi, F.X. Farrell, A. Bittner, J.E. Galindo, M. Morton, H. Trinh, L.K. Jolliffe, Erythropoietin induces changes in gene expression in PC12 cells, Brain Res. 104 (2002) 86–95.
[79] D. Ribatti, M. Presta, A. Vacca, R. Ria, R. Giuliani, P. Dell'Era, B. Nico, L. Roncali, F. Dammacco, Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo, Blood 93 (1999) 2627–2636.
[80] W. Risau, Mechanisms of angiogenesis, Nature 386 (1997) 671–674.
[81] K. Ruscher, D. Freyer, M. Karsch, N. Isaev, D. Megow, B. Sawitzki, J. Priller, U. Dirnagl, A. Meisel, Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model, J. Neurosci. 22 (2002) 10291–10301.
[82] Y. Sadamoto, K. Igase, M. Sakanaka, K. Sato, H. Otsuka, S. Sakaki, S. Masuda, R. Sasaki, Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery, Biochem. Biophys. Res. Commun. 253 (1998) 26–32.
[83] M. Sakanaka, T.C. Wen, S. Matsuda, S. Masuda, E. Morishita, M. Nagao, R. Sasaki, In vivo evidence that erythropoietin protects neurons from ischemic damage, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 4635–4640.
[84] S.T. Sawyer, S.B. Krantz, K. Sawada, Receptors for erythropoietin in mouse and human erythroid cells and placenta, Blood 74 (1989) 103–109.
[85] F. Schba, A.Y. Schwartz, I. Chereshnev, J. Bederson, Acute decrease in cerebral nitric oxide levels after subarachnoid hemorrhage, J. Cereb. Blood Flow Metab. 20 (2000) 604–611.
[86] S. Sela, R. Shurtz-Swirski, R. Sharon, J. Manaster, J. Chezar, G. Shkolnik, G. Shapiro, S.M. Shasha, S. Merchav, B. Kristal, The polymorphonuclear leukocyte—a new target for erythropoietin, Nephron 88 (2001) 205–210.
[87] T. Shingo, S.T. Sorokan, T. Shimazaki, S. Weiss, Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells, J. Neurosci. 21 (2001) 9733–9743.
[88] A.D. Sinor, D.A. Greenberg, Erythropoietin protects cultured cortical neurons, but not astroglia, from hypoxia and AMPA toxicity, Neurosci. Lett. 290 (2000) 213–215.
[89] A.L. Siren, H. Enrenreich, Erythropoietin—a novel concept for neuroprotection, Eur. Arch. Psychiatry Clin. Neurosci. 251 (2001) 179–184.
[90] A.L. Siren, M. Fratelli, M. Brines, C. Goemans, S. Casagrande, P. Lewczuk, S. Keenan, C. Gleiter, C. Pasquali, A. Capobianco, T. Mennini, R. Heumann, A. Cerami, H. Ehrenreich, P. Ghezzi, Erythropoietin prevents neuronal apoptosis after cerebral ischemia and in metabolically stressed neurons, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 4044–4049.
[91] A.L. Siren, F. Knerlich, W. Poser, C.H. Gleiter, W. Bruck, H. Ehrenreich, Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain, Acta Neuropathol. 101 (2001) 271–276.
[92] I. Solaroglu, A. Solaroglu, E. Kaptanoglu, S. Dede, A. Haberal, E. Beskonakli, K. Kilinc, Erythropoietin prevents ischemia-perfusion from inducing oxidative damage in fetal rat brain, Child Nerv. Syst. 19 (2003) 19–22.
[93] J.B. Springborg, X. Ma, P. Rochat, G.M. Knudsen, O. Amtorp, O.B. Paulson, M. Juhler, N.V. Olsen, A single subcutaneous bolus of erythropoietin normalizes cerebral blood flow regulation after subarachnoid haemorrhage in rats, Br. J. Pharmacol. 135 (2002) 823–829.
[94] L. Studer, M. Csete, S.H. Lee, N. Kabbani, J. Walikonis, B. Wold, R. McKay, Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen, J. Neurosci. 20 (2000) 7377–7383.
[95] M. Sugawa, Y. Sakurai, Y. Ishikawa-Ieda, H. Suzuki, H. Asou, Effects of erythropoietin on glial cell development; oligodendrocyte maturation and astrocyte proliferation, Neurosci. Res. 44 (2002) 391–403.
[96] T. Tabira, Y. Konishi, F. Gallyas, Neurotrophic effect of hematopoietic cytokines on cholinergic and other neurons in vitro, Int. J. Dev. Neurosci. 13 (1995) 241–252.
[97] M. Vairano, C.D. Russo, G. Pozzoli, A. Battaglia, G. Scambia,

G. Tringali, M.A. Aloe-Spiriti, P. Preziosi, P. Navara, Erythropoietin exerts anti-apoptotic effects on rat microglial cells in vitro, Eur. J. Neurosci. 16 (2002) 584–592.

[98] P. Villa, P. Bigini, T. Mennini, D. Agnello, T. Laragione, A. Cagnotto, B. Viviani, M. Marinovich, A. Cerami, T.R. Coleman, M. Brines, P. Ghezzi, Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis, J. Exp. Med. 198 (2003) 971–975.

[99] M.R. Wald, E.S. Borda, L. Sterin-Borda, Mitogenic effect of erythropoietin on neonatal rat cardiomyocytes: signal transduction pathways, J. Cell Physiol. 167 (1996) 461–468.

[100] A. Weber, R.F. Maier, U. Hoffmann, M. Grips, M. Hoppenz, A.G. Aktas, U. Heinemann, M. Obladen, S. Schuchmann, Erythropoietin improves synaptic transmission during and following ischemia in rat hippocampal slice cultures, Brain Res. 958 (2002) 305–311.

[101] T.C. Wen, Y. Sadamoto, J. Tanaka, P.X. Zhu, K. Nakata, Y.J. Ma, R. Hata, M. Sakanaka, Erythropoietin protects neurons against chemical hypoxia and cerebral ischemic injury by up-regulating Bcl-xL expression, J. Neurosci. Res. 67 (2002) 795–803.

[102] C. Wiessner, P.R. Allegrini, D. Ekatodramis, U.R. Jewell, T. Stallmach, M. Gassmann, Increased cerebral infarct volumes in polyglobulic mice overexpressing erythropoietin, J. Cereb. Blood Flow Metab. 21 (2001) 857–864.

[103] H. Wu, S.H. Lee, J. Gao, X. Liu, M.L. Iruela-Arispe, Inactivation of erythropoietin leads to defects in cardiac morphogenesis, Development 126 (1999) 3597–3605.

[104] R. Yamaji, T. Okada, M. Moriya, M. Naito, T. Tsuruo, K. Miyatake, Y. Nakano, Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA, Eur. J. Biochem. 239 (1996) 494–500.

[105] M. Yamamoto, K. Koshimura, M. Kawaguchi, M. Sohmiya, Y. Murakami, Y. Kato, Stimulating effect of erythropoietin on the release of dopamine and acetylcholine from the rat brain slice, Neurosci. Lett. 292 (2000) 131–133.

[106] Y. Yasuda, S. Masuda, M. Chikuma, K. Inoue, M. Nagao, R. Sasaki, Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis, J. Biol. Chem. 273 (1998) 25381–25387.

[107] A. Yoshimura, H. Misawa, Physiology and function of the erythropoietin receptor, Curr. Opin. Hematol. 5 (1998) 171–176.

[108] X. Yu, C.S. Lin, F. Costantini, C.T. Noguchi, The human erythropoietin receptor gene rescues erythropoiesis and developmental defects in the erythropoietin receptor null mouse, Blood 98 (2001) 475–477.

[109] X. Yu, J.J. Shacka, J.B. Eells, C. Suarez-Quian, R.M. Przygodzki, B. Beleslin-Cokic, C.S. Lin, V.M. Nikodem, B. Hempstead, K.C. Flanders, F. Costantini, C.T. Noguchi, Erythropoietin receptor signalling is required for normal brain development, Development 129 (2002) 505–516.